THESIS

ON

STUDY OF METABOLISM IN BONE WITH RADIOACTIVE PHOSPHORUS AND BY AUTORADIOGRAPHY

SUBMITTED FOR THE DEGREE OF

Ph.D.

by

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HISTORICAL AND INTRODUCTORY

A. HISTORY OF RADIOACTIVE ISOTOPES

Ever since the discovery of radioactivity by Becquerel in 1896 and the subsequent isolation of pure radium by Madame Curie in 1899, the study of radioactivity in its manifold aspects and diverse applications has been carried out by various scientists in all parts of the interested world and more work has been done on nuclear physics during the last half century than on any other particular branch of physical science.

The study of atomic research started with Lord Rutherford who, in 1911, while working with the radioactivity of certain elements, reached the stage where he was confronted with the authenticity of the definition of an atom, and found that the truth of his experimental findings refused to admit the original Greek definition of an atom (ἄτομος = indivisible). This was a challenge thrown against the most ancient conception of the origin of matter first conceived by the visionary eyes of ancient Hindu philosophers nearly 2000 B.C. and which had hitherto successfully stood the acid test of time through centuries, having the approval of such astute thinkers as Aristotle, Democritus and Epicurus in the Middle Ages, and Dalton, Lavoisier and Faraday, of comparatively recent /

recent years.

An atom had hitherto been considered as the ultimate, indivisible and indestructible particle of matter which is stable and of uniform size differing only in chemical behaviour in different elements but Rutherford had to extend further this definition of an atom and being convinced by his experimental observations postulated in 1912 the modern conception of an atom. He revealed that an atom of an element is not a stable indivisible particle but is a conglomeration of electrical charges. According to him, an atom structurally consisted of a central positively-charged nucleus which is counterbalanced by an equivalent negative charge of electrons revolving round the nucleus in their own orbits. Various analogies have been summoned to hazard a clearer mental picture of an atom. Thus it has been aptly compared with the solar system where the sun is compared with the central nucleus and the planets with electrons revolving round the periphery of the nucleus. The present view is that the electrons form a "cloud" (electron cloud) around the nucleus, of total charge equal in magnitude to the positive nuclear charge, the atom as a whole being electrically neutral. This cloud has a structure corresponding roughly to the old electron orbits but work on the wave properties of the electron forced the abandonment of the idea of definite electron orbits. To

appreciate /

appreciate the infinitesimally small diameter of an atomic orbit it has been mentioned that the average man's height stands midway between the diameter of the solar system and that of an atom, i.e., "while the former is about one hundred thousand million times greater than a man's height, the latter is about one hundred thousand millionth as large as a man" (Aebersold, 1942). The size of an atomic nucleus is "about one million millionth part of a centimeter in diameter and about ten thousand times smaller than an atomic diameter" (Aebersold, 1942). The number of atoms in one cubic centimeter of air which is quite tenuous is about ten billion billion, which is about the number of red blood cells in one million people. About half the number of electrons, however, are in such motion around the periphery of the nucleus, while the other half lie in intimate combination with protons at the centre. These latter combined electrons and protons are called "neutrons" since they are neutral to an electro-magnetic field. Further research into the structure of an atom revealed that the mass of an atomic nucleus is far too heavy for an electronic mass and as such "the atomic mass" of an element is taken as the mass of its central nuclear bulk of protons and neutrons and denoted by the letter 'A', while "the atomic number" denotes the number of revolving electrons and is indicated by the letter 'Z'. Further, "the atomic number" of an element is constant and hence atoms /

atoms of the same atomic number are necessarily atoms of the same element. In all chemical reactions between different elements it is the peripheral electrons which take part in the interchange forming compounds leaving the nucleus quite unaffected.

Later, it was also found that the same element can exist in nature in different forms maintaining, at the same time, its basic parent nature - "like different varieties of oranges" (Aebersold, 1942). Such an apparent contradictory combination in an element can only be structurally explained, and has been proved by its having the same atomic number with a different atomic mass and such a variation of an element having the same atomic number with a different atomic mass is called an "isotope" of that element (ἴσος = same; τόπος = place). The first experimental evidence of isotopy was obtained by Boltwood in 1905 (Boltwood, 1907), though it was not understood at the time, when he discovered radioactive ioniom which is an isotope of thorium. The discovery of non-radioactive isotopes was, however, made at a later date by Thompson who, in 1907 (Thompson, 1912) first proved by experiments the existence of two isotopes of the element neon.

In nature, the isotopes of comparatively lighter elements are stable, while those of the relatively heavier ones, owing to their nuclear imbalance between protons and neutrons, are constantly undergoing disintegration and as such, are radioactive.

This /

This process of nuclear disintegration goes on until, after a varying period of time, ranging from fractions of an hour to thousands of years and during which one or more intermediate radioactive products may be produced, the initial unstable element settles into another stable one above or below in the periodic table, according to whether it loses an electron or an α-particle during its process of disintegration. Thus, the element uranium, after forming eventually such intermediate radioactive elements as ionium and radium, is finally converted into stable lead. Similarly, radioactive phosphorus is converted into stable sulphur, beryllium into boron, and so on.

The first study of applied radioactive isotopy was made by Hevesy in 1912 (Hevesy, 1923) when he used radioactive isotopes as "tracers" in chemical investigations. The "tracer" study in biological investigations means tracing or following the biological behaviour of any element in the plant, animal or human system by "tagging" or "ear-marking" the element in question with small, innocuous doses of the radioactive isotope. This latter, by dint of its radioactivity and chemical identity with the element to be traced, offers a very suitable help for the detection of the former at any stage throughout its biological course. Thus, the absorption, deposition in tissues and elimination of any element of biological importance, can be studied in detail by tagging it with a suitable radioactive isotope. /

isotope. The method was first extended to biological studies by Hevesy in 1923, when he employed radioactive lead (radium D) to investigate the uptake and distribution of this element in plants but owing to the lack of naturally occurring isotopes of the biologically important elements, the use of this powerful investigative tool remained limited until a method of preparing isotopes artificially was discovered in 1934 by Irene Curie and Professor Joliot, her husband. They discovered that when the nucleus of an aluminium atom is bombarded with α-particles from a natural radioactive source, the latter can be made to break through the potential barrier of the former, producing the following nuclear reaction:

$$13A^{27} + {}_2He^4 \rightarrow 15P^{30} + {}_0n^1 \quad \text{(neutron)}$$

P^{30} is a radioactive isotope of phosphorus emitting positrons with a half-life of 195 seconds. Later, a more effective substitute for ordinary α-particles was found in heavy hydrogen or deuterium, the nucleus of which is designated as deuteron, but in spite of its greater effectiveness than an ordinary α-particle, deuterons had to be accelerated to high speeds artificially, since they are not a natural product of radioactivity. Investigators were now faced with the difficulty of obtaining high-speed deuterons for a more severe bombardment of the relatively heavier elements. /

elements. This difficulty was, however, soon obviated through the genius of Lawrence (1941) and of Lawrence and Livingston (1932) of the University of California who, by the invention of the cyclotron in 1930, paved the path for a rapid progress in the use of radioactive isotopes, especially in biological investigations, and quite a number of them have since been effectively carried out.

B. HISTORY OF AUTORADIOGRAPHY /

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Principle

When a piece of plant, animal or human tissue containing a radioactive isotope is brought in contact with a specially sensitive photographic emulsion, the radiations from the isotope activate the emulsion in the same way as light rays act on an ordinary photographic plate, and on subsequent processing enables us to have a self-portrait or an "autoradiogram" of the said tissue, localising the area which incorporated the isotope.

History

The history of autoradiography is almost as old as photography. Shortly after the acceptance of silver halide emulsion as a recording medium for optical images, Moser (1842) observed that many non-metallic substances would affect a photographic emulsion in the dark and produce an image on subsequent processing. This observation was soon followed by Niepce de Saint-Victor (1867) who observed the fogging of silver halides by different uranium compounds but attributed it to simple luminescence phenomena and thus missed the first observation on autoradiography. In 1896, Becquerel repeated Niepce's experiment and found that the activation of the silver halide emulsion by uranyl sulphate /

sulphate was due to the radioactive property of the latter and this observation was an important adjunct to his discovery of radioactivity. Following this principle, Lacassagne and Lattes (1924) finally demonstrated the distribution of radio-elements in different organs of animals by injecting them with radioactive compounds and exposing the paraffin tissue blocks on photographic emulsions. Lacassagne it was who introduced the term "autoradiogram" to these self-portraits of radioactive tissues.

Preparation of an Autoradiogram

In making an autoradiogram, the radioactive substance is first of all introduced into the animal parenterally or intravenously and time allowed for it to be deposited in the desired organ. The animal is then sacrificed and a thin section of the tissue is brought in contact with the photographic emulsion. The first, essential point to be observed while making an autoradiogram, is an intimate and firm contact between the autoradiographic plate and the specimen to be autoradiographed as the intensity of the image varies inversely to the square of the distance between the plate and the source of radiation. It is all the more necessary considering the different tissue-penetration properties of radio-elements emitting β-particles.

A number of methods have been described for this purpose and most of them are individual variations /

variations of the principle of bringing the object in intimate contact with the photographic emulsion previous to exposure and processing. Some workers (Belanger and Leblond, 1946; Gross et al., 1951) have done it by painting an ordinary slide with a thin coating of the photographic emulsion and bringing the specimen containing radioactive isotope in contact with it before exposing and processing it. Others, e.g., Evans (1947), have used the same technique in a reverse order by pouring the emulsion on the slide with the specimen on it. In both these methods the adequate amount of emulsion required for a successful autoradiogram has to be found out by trial and error and manual dexterity is necessary in order to obtain a normal autoradiogram. To obviate this difficulty the "stripping film technique" developed by Pelc (1949) (described later) has been used by many workers using isotopes of water-insoluble elements.

For an autoradiogram of a section of bone, the following points should be observed:-

(1) The thickness of the section should be as near to the thickness of a single osteocyte (4.8 microns) as possible, as the greater radiation coming from a thicker section blurs the outline of the section in the autoradiogram.

(2) The amount of radioactive isotope deposited in the bone should be sufficient for even its thinnest parts to activate the grains of the photographic emulsion, preferably within the half-life of the isotope./

isotope.

(3) The speed of the photographic plate should enable one to have a successful autoradiogram with an exposure not exceeding the half-life of the isotope.

(4) The developing and fixing solutions should be of reliable chemical purity in order to avoid artefacts indistinguishable from developed grains during subsequent microscopical examination of the autoradiogram. The solutions should preferably be prepared in the laboratory and stored in amber bottles leaving no column of air on top in order to avoid decomposition.

(5) The films should be developed at a temperature of 18.5°C., as any temperature on either side of it prevents complete resolution of grains within the optimum time for development or fogs the background.

"Stripping Film Technique" of Pelc

Kodak special autoradiographic plates are used in this technique. These are supplied with the photographic emulsion spread over a thin layer of gelatin to allow for an easy stripping of the emulsion off the glass plate.

A rectangular piece of the film, enough to cover the specimen on the slide, is cut with a sharp scalpel and stripped off the glass plate. It is then reversed and floated on the surface of water contained in /

in a shallow dish so that the emulsion-side looks down. The film is given about three minutes to swell, when the slide with the section is passed under it and lifted off the water so that the film spreads evenly on the surface of the slide covering the specimen. A little tilt on either side allows the redundant portions of the film to adhere on the reverse surface of the slide and thus ensures a firm contact between the specimen and the film. The slide is then dried and exposed in the dark. The final processing is subsequently done following the photographic principle, with a modified temperature of the developing solution (18.5°C.).

We have obtained satisfactory results using the above technique with the slight modification that water was replaced by absolute alcohol, as the former dissolves P^{32}. In addition, we have developed a technique which enabled us to avoid the use of liquids during mounting. The technique is described as follows:

Eastman Kodak nuclear track plate is used for this purpose. An area of about $1\frac{1}{2}$" in length and $\frac{3}{4}$" in width is cut with a sharp scalpel and stripped off the plate and floated on the surface of a shallow enamel dish (6" x 4" x 1") containing redistilled water up to about three quarters of its depth. The gelatin surface of the film rests on the surface of the water. A perspex slide, previously cleaned in a /

a solution of potassium bichromate and sulphuric acid is gently passed under the film and quickly lifted up with the film evenly spread out over it. The surface tension exerted by water allows for a firm contact between the film and the slide and the mounted slide is then dried in front of an electric fan.

When the film is perfectly dry, it is brought into close contact with the specimen in the following way (Fig. 47):-

An ordinary photographic contact frame is taken. The slide containing the film is placed on the glass of the frame, keeping the emulsion-side up and the slide containing the thin ground bone-section is then laid against it so as to bring the specimen in intimate contact with the emulsion. The wooden cover is then carefully laid on and tightly screwed. The whole procedure is obviously carried out in the dark-room. A safe light is used but at a distance not less than two feet from the working table, as a distance less than that, has fogged some of our films. The mounted film is then put in a light-tight box and kept in a refrigerator and exposed for twelve days at 4°C.

It will be noted that throughout the whole procedure, we have avoided any water coming in contact with the specimen and thus preserved the integrity of the calcium phosphate molecules in the specimen.

Processing /

Processing. - The exposed autoradiographic film is developed in the following special developing solution at 18.5°C:-

Mettol	-	4 g.
Sodi. sulph. cryst.	-	300 g.
Hydroquinone	-	16 g.
Sodi. carb. cryst.	-	200 g.
Pot. brom.	-	10 g.
Redistilled water ad	-	2000 ml.

Each ingredient should be dissolved in order, in warm, redistilled water, and allowed to cool before use. The film is developed for from 5 - 8 minutes depending on the region of the bone for visualisation. Regions containing greater amount of bone tissue, e.g., ends of a long bone, show up quicker on the autoradiogram than regions containing lesser amount, owing to the greater deposition of radio-element in the former. In order to develop the whole length of a rat femur, an average time of six minutes is sufficient. The developed film is then cleared and fixed by a special fixing and clearing solution of the following composition:-

F53 (AnalaR Chemicals)

Sodi. sulph. cryst.	-	100 g.
Glacial acetic acid	-	75 g.
Pot. alum.	-	100 g.
Redistilled water ad	-	1000 ml.

F54A (AnalaR Chemicals)

Warm redistilled water	-	500 ml.
Sodi. thiosulph. cryst.	-	400 g.

Allow solution F54A to cool and then add 18.5 ml. of F53 /

F53 solution to it and make it up to 1000 ml. with cold redistilled water. The solution should not be kept for more than a fortnight before use and it should not be used if any discolourisation or precipitate appear in the course of preparation or keeping. Finally, the film is washed in running, distilled water, dried and examined under a phase-contrast microscope. Purity of chemicals in the developing and fixing solutions is essential, as otherwise, artefacts indistinguishable from developed grains are likely to appear and vitiate the whole procedure. The temperature of the developing solution is also critical as any temperature above or below 18.5°C. either fogs the field or does not allow maximum resolution of grains within the optimum time for development.

REVIEW OF LITERATURE /

REVIEW OF LITERATURE

In order thoroughly to understand the action of radioactive phosphorus on bone one should have systematic observation on undifferentiated mesenchymal bone as well as on various stages of development and under different physiological conditions. But in reviewing the literature dealing with the uses of radioactive phosphorus, one unfortunately finds that, in comparison with its experimental and therapeutic uses in other systems, the literature dealing with the action of radioactive phosphorus on bone, is limited. However, of the more than five hundred isotopes discovered hitherto of which about twelve have been studied biologically, radioactive phosphorus has been the most extensively applied, because from the discovery of artificial radioactivity to the present, it has been the most readily available.

A. ACTION OF RADIOACTIVE PHOSPHORUS ON BONE /

A. ACTION OF RADIOACTIVE PHOSPHORUS ON BONE

Before the discovery of radioactive isotopes and their subsequent use in various biological investigations, the study of metabolism of phosphorus in bone was mostly deduced in an indirect manner from its general metabolism in the body. The chief reason for this was the lack of a suitable tool for direct observation on bone. In so far as the general metabolism of phosphorus is concerned, various works have been published on the storage and elimination of phosphorus in various tissues (Cheiwitz and Hevesy, 1935; Scott and Cook, 1937; Cook et al., 1937), the rate of phospholipid synthesis (Artom et al., 1937; Lundsgaard and Hevesy, 1937; Hahn and Hevesy, 1937) and also upon the site of this synthesis (Parlman et al., 1937).

Prior to 1935, studies on the metabolism of phosphorus were carried out by such means as the use of intestinal fistulae, addition of an inactive, unabsorbed marker to the food, such as iron oxide as used by Bergeim (1926) or by means of differential analysis of faecal pellets, in order to demonstrate the changes in the concentration of calcium and phosphorus from caecum to anus. The discovery of radioactive isotopes provided us with a very suitable tool for direct study on bone itself and without the necessity of demineralisation.

Hevesy /

Hevesy, in 1923, demonstrated the use of radioactive isotopes as indicators in biological investigations and Cheiwitz and Hevesy (1935) were the first to use tracer doses of radioactive phosphorus in the study of bone metabolism. They fed rats with radioactive phosphorus and, after some days, estimated the deposition by a Geiger-Müller counter and showed that in the adult rat, about 30 per cent of the phosphorus was deposited in the skeleton and removed completely from the body in twenty days.

A number of works have since been published on direct observations on bone metabolism with the aid of radioactive compounds, but in the majority, these have been made only quantitatively, either by Geiger-Müller counter or by biochemical means.

Regarding the absorption and deposition on bone of radioactive phosphorus, various workers have come to more or less the same conclusions working on different kinds of animals. Cohn and Greenberg (1939) determined the absorption of phosphorus in rat bone and found that the major deposition of injected or ingested phosphate occurred within eight hours after administration, and 20 - 30 per cent of the absorbed phosphorus is excreted with urine within eight hours. Hahn et al. (1937) made a more thorough study of the behaviour of radioactive phosphorus in bone and found that it takes twenty days for injected or ingested radioactive phosphorus to be completely excreted from the bone. About 30 per cent /

cent of the administered radioactive phosphorus is deposited in bone of which again only one-tenth takes part in the maintenance of the dynamic equilibrium, while the rest is "protected" and not released into the blood stream within a month. This observation has been further confirmed by Hevesy (1937) and by Hevesy and Hahn (1944). Manly et al. (1940) found that the total amount of blood phosphorus is not simultaneously deposited in bone. They have differentiated a "labile fraction" which is probably similar to the fraction for dynamic equilibrium as found by Hevesy and Hahn (1944) and a "stable fraction" which starts to accumulate from the fifth day onwards after its administration. Manly and Bale (1939) observed that about twice the amount of injected radioactive phosphorus is deposited in the epiphysis of long bones at the end of the first day. Pêcher (1942) made a comparative estimation of the distribution of radioactive phosphorus and found that the largest fraction is deposited in bone. Greenberg (1945) observed that the level of radioactive phosphorus in bone begins to rise within half an hour after injection and attains a peak of 30 per cent at the eighth hour. The time taken by a rabbit's body tissues to eliminate completely the radioactive phosphorus is, according to him, one month, which agrees with Hevesy's findings. Neuman and Riley (1947) conclude from their experiment in bone that it is rather the increased general circulation than any specific /

specific activity of the growing osteocytes which is responsible for the deposition of radioactive phosphorus in experiments of short duration. Our own observations on the absorption and deposition of radioactive phosphorus on bone are in line with the mentioned findings. The difference in the elimination-time between our finding (see below) and that of Hahn et al., (1937) can only be explained by assuming an increase of seventy-two hours in the elimination of the injected isotope in rats as used in our experiments from that in rabbits. Manly and Bale (1939) followed the line of investigation of Falkenheim et al. (1947) and came to similar conclusions regarding adsorption and desorption of P^{32} in bone.

In induced rachitic animals, not only the uptake of radioactive phosphorus is increased but there is also a significant rise in the level of concentration and the rate of deposition in bone. Administration of vitamin D further promotes those actions both in vivo and in vitro. Copp and Greenberg (1943) mention that the action of vitamin D is mainly to convert organic phosphorus to inorganic phosphorus in bone and it has no effect on the transference of organic phosphorus from blood to bone (supported by Shimotori and Morgan, 1947). He also maintains that in rickets, in view of the low phosphate content in plasma and presence of glycogen in it, the transference of organic phosphorus from blood is preceded by /

by its formation in plasma by the action of phosphorylase and phosphoglucomutase converting the plasma glycogen and phosphate into glucose-1-phosphate and glucose-6-phosphate respectively.

Riley et al. (1945) found no difference in the deposition of P^{32} in normal and denervated bone. Greenberg (1945) showed that vitamin D promotes the absorption of calcium and strontium in bones and that vitamin D has a direct action on its mineralisation.

Gunther et al. (1943) followed in detail one case of primary human rickets and concluded that the primary cause of rickets in a child is not failure of absorption of calcium but rather failure of the calcifying mechanism.

B. AUTORADIOGRAPHY /

B. AUTORADIOGRAPHY

The literature dealing with autoradiography extends over a considerably large and varied field and specimens widely differing in both physical and chemical nature have been subjected to autoradiography by different workers.

Polished metal surfaces, e.g., copper-ore sections, have been autoradiographed by Yagoda (1949). Copper sulphate crystals ($CuSO_4,5H_2O$) embedded in wax and supported by a screw-cap frame, have also been similarly autoradiographed by the same worker.

In the plant kingdom, also, successful autoradiograms have been prepared with sections of various elements, e.g., tomato plant and fruit (Stevens, 1950), cotton, willow and geranium plants (Stout et al., 1940), barley plants (Scott-Russell et al., 1949), bean root (Pelc, 1950). Stout (1941) also demonstrated by autoradiography that, contrary to the older theory of the ascent of sap in plants, there is also a downward rhythmic movement in them.

Various zoological specimens have been autoradiographed by workers in this field extending over a variety of objects, e.g., larvae of silk-producing moths (Hamilton, 1942), Paramaecium and dogfish (Harris and King, 1950), mosquitoes (Hassett and Jenkins, 1949). In the animal kingdom, Pelc and Howard /

Howard (1950) made autoradiograms of the chromosome of meiotic cells from rat testes. Hamilton (1942) prepared the same of animal lung tissue with aerosols of plutonium oxide. Chick fibroblasts in tissue culture have been impregnated with radio-active phosphorus and autoradiographed by Pelc (1949).

Regarding the methods of autoradiography in animal tissues, Belanger and Leblond (1946) prepared by painting the photographic emulsion on the specimen while Evans (1947) obtained successful autoradiograms by a reversed method, i.e. by immersing the specimen in the emulsion. Holt et al. (1949) and Harris and King (1950) used the freeze-drying technique in making autoradiograms of animal and human tissues. The stripping film technique of Pelc (1949) is by far the most accepted method of autoradiography today and it is recognised as an established method of technique by a number of workers (Bayley, 1947; Boyd and William, 1948). Winteringham's (1950) dry method obviates the loss of water soluble isotope resulting from other techniques.

STUDY OF METABOLISM OF PHOSPHORUS IN BONE BY RADIOACTIVE PHOSPHORUS /

STUDY OF METABOLISM OF PHOSPHORUS IN BONE BY RADIOACTIVE PHOSPHORUS

The purpose of the present work was to correlate the quantitative observations of phosphorus metabolism in bone by P^{32} with corresponding qualitative observations by autoradiography. The plan of work was as follows:-

Experiment 1.- Observations on the normal metabolism of phosphorus in undemineralised bone.

Experiment 2.- Observations in artificially-induced rickets.

Experiment 3.- Observations on the effect of parathyroid extract on bone.

In each item of the above series of experiments, the quantitative estimation of phosphorus was determined by the Geiger-Müller counter and the bones were subsequently autoradiographed for qualitative examination.

Procedure

Experiment 1.- Fifty healthy rats six to eight weeks old and between 180 and 250 g. in weight, were maintained on a uniform normal diet. The animals were injected intravenously or intraperitoneally with 1 ml. of P^{32} as sodium orthophosphate compound. The amount contained in 1 ml. varied between 150 to 300 microcuries. The first twenty-eight animals in Experiment /

Experiment 1 were injected intravenously by open venesection, while the remainder by the closed method, after having previously transilluminated the veins by an apparatus similar to the one described by Nickson and Barkulis (1947). Animals were killed at hourly intervals following injection, sacrificing four at a time, and of the two left over, one was killed after twenty-four hours and the other after forty-eight hours, respectively. Both femurs from each animal were dissected out, freed of soft tissues and dried in the incubator at 45°C. for three hours. After taking the individual dry weights and ashing them separately by the technique to be described later (page 37), each of the one hundred specimens of femur was counted for radioactivity in the Geiger-Müller counter. After removing the background error, a two-minute count for each specimen was made and the calculated mean taken as the amount of radiation per minute for each femur. Ten such counts were taken for each bone and from them and their over-all count, the standard deviation for each bone was calculated. This procedure was repeated in Experiments 2 and 3 of the series and the results were tabulated and plotted into curves.

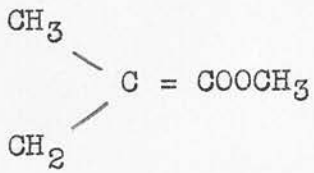
After recording the total count, each bone was autoradiographed. In order to obtain a satisfactory autoradiogram of bone containing radioactive phosphorus one should endeavour to have the maximum effect of the energy contained in these β-particles on /

on the photographic emulsion and for this purpose it is imperative to have the tissue in a thin section, preferably below eight microns in thickness. The chief difficulty encountered was to obtain a thin enough section of undecalcified bone. In auto-radiography of bone with P^{32}, decalcification cannot be employed, since it causes loss of phosphorus and no ordinary microtome is capable of making sections thin enough when undecalcified bone is merely embedded in such media as paraffin, which is the most common embedding medium for soft-tissue sections. In order to maintain the density of the embedding medium as near to that of the specimen as possible, so as to buffer the momentum of the microtome knife and thus prevent crumbling of the bone during the making of a thin section, different kinds of plastic compound have been used by different workers.

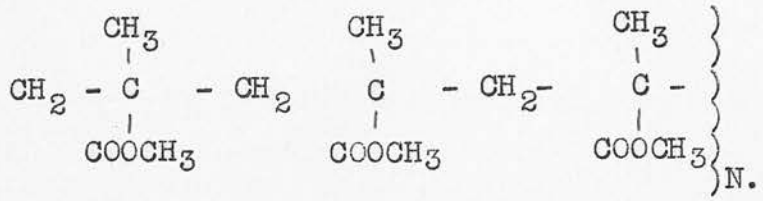
As a chemical substance, plastic or methyl methacrylate has been known to the chemical world for a long time. It is a colourless liquid with a boiling point of 100.3°C. which is converted into a clear solid when exposed to sunlight or when heated. Liquid methyl methacrylate is a monomeric substance, whereas the clear solid which is produced from it by the action of heat or light is called a polymeric substance or a polymer. The chemical process by which /

which a monomer is converted into a polymer is as follows:-

Monomer



Polymer



The variety of patent plastic or methyl methacrylate and allied preparations offered by different manufacturers is legion, and of these, nitrocellulose, celluloid, kallodent, stellon and kallodoc liquid, are the chief ones used for embedding hard tissues like bone or teeth (Holt, 1948a, b). We have used the last-named preparation for our work (Puckett, 1941).

Preparation of the Monomer

The methyl methacrylate preparation - "kallodoc liquid"*- contains a stabiliser in order to prevent spontaneous polimerisation. This stabiliser is first of all removed by washing the monomer with an equal volume of 5 per cent caustic soda solution, followed /

*Literature obtainable from the Plastic Division, Imperial Chemical Industries, Limited, Welwyn, U.K.

followed by extraction with distilled water until the washings are no longer alkaline. Washing is best carried out in a separating funnel when the methyl methacrylate monomer forms the upper of the two layers. The washed monomer is then allowed to stand for twenty-four hours over anhydrous calcium chloride. After filtration of the same, it is ready for use and stored in a refrigerator and the flask labelled Flask A. Another portion of the kallodoc freed from the stabiliser in the above way is taken into Flask B and to it 0.1 - 0.5 per cent of benzoyl peroxide is added and similarly stored. Immediately before use, the methyl methacrylate monomer from Flask A and Flask B is taken in a third flask, Flask C, in proportions of ten parts of the former to one part of the latter and heated in a water-bath at a temperature of 37°C. This temperature of polimerisation is critical as any temperature above 37°C. is sure to trap air bubbles around the specimen which hinder the subsequent making of thin sections. A lower temperature on the other hand, delays polimerisation and considering the 14.3 days half-life of P^{32}, it does not leave much time for the more elaborate and time-consuming procedure that follows embedding. The flask is kept stoppered and constantly rocked in the water-bath with a gentle to-and-fro movement. Every ten minutes the flask is taken out of the bath and /

and uncorked so as to drive out the accumulated air bubbles. The liquid is heated in this manner until it attains a syrupy consistence. The optimum consistence has to be found out by trial and error and this also is critical as any consistence on either side of the optimum is likely either to produce spontaneous polimerisation or prolong the polimerisation-time to beyond the half-life of P^{32}. For our purpose, we have obtained the optimum consistence by heating the methyl methacrylate monomer for twenty-two minutes in the above manner under normal atmospheric pressure. With this consistence it usually takes six to eight hours for polimerisation to be complete at a temperature of 37°C.

After thus preparing the monomer, and before actually mounting the specimen of bone on it, a preliminary platform is made by filling a small test tube ($3\frac{1}{2}$" in length and $\frac{1}{4}$" in diameter) with the prepared methyl-methacrylate solution to a depth of about $\frac{3}{4}$" and allowed to polimerise slowly in the incubator. The test tube is brought out of the incubator just a little before complete polimerisation so as to allow the bone to be implanted into it manually and without much resistance. The test tube is now held vertically by means of a holder fitted on to a stand and the femur gradually inserted upright into the semi-solid platform with a firm and even pressure. Care should be taken to avoid tilting of the bone produced by uneven pressure during /

during implantation. Some workers have preferred boring a hole into a completely solidified platform and subsequently position the bone erect into it, but we have found this procedure hazardous, as it is difficult to keep the diameter of the hole equal to that of the bone, owing to the shrinkage of the former during final polimerisation and also on account of the air bubbles that result from the subsequent filling-up of the test tube with a further amount of methyl methacrylate solution.

Having thus positioned the femur on the semi-solid platform, the test tube is then filled up completely with a further amount of monomer and allowed to polimerise completely in the incubator. The importance of keeping the consistence of the monomer to the optimum and allowing it to polimerise slowly at 37°C. comes at this stage, as any consistence on either side of the optimum is likely either to trap air bubbles coming from the connective tissue or prolong the time for complete polimerisation to beyond the half-life of P^{32} (Fig. 7).

After the polimerisation is complete, the specimen is brought out of the incubator and the test tube broken off so as to leave behind a clear, solid block of monomer with the bone embedded in it. It is then cut into suitable smaller blocks by means of a circular saw driven by a motor and shaped into cubes of about one cubic millimeter in size by grinding /

grinding the sides against a revolving carborundum wheel. One of these blocks is then mounted on the microtome for sectioning.

In order to make a thin section of an undecalcified bone, it is necessary for any microtome knife serving the purpose to combine heaviness with adequate hardness and sharpness so as to allow for both momentum and precision. Axelrod (1947) finds the "Spencer" type of heavy microtome suitable for this purpose, and with it she has been able to obtain bone sections even up to six microns in thickness. Others have ground the bone specimens and obtained even thinner sections of undecalcified bone or teeth, although the time taken by them has been naturally long. Thus, to obtain a tooth section ground to a thickness of four microns, it took Duckworth (1950) twelve to fourteen hours and Leblond (Belanger and Leblond, 1946) does not mention the time taken by himself. We have combined the above two methods in our procedure, firstly because the thinnest section obtainable from the microtome at our disposal is as thick as one millimeter and, secondly, by combining these two methods the time taken for subsequent grinding has been shorter. The heavy sledge precision microtome (Fig. 8) was found to serve the purpose best, and with it a one millimeter section of the specimen could be obtained. This one millimeter /

millimeter-thick section is then smeared on one surface with a thin layer of chloroform, enough to dissolve partially the area of perspex surrounding the bone. The specimen is then gently put on a perspex slide and the whole preparation incubated at 45°C. for about twenty minutes, at the end of which the embedded specimen adheres firmly to the slide. Glass slides are of no use since they do not adhere to the perspex enough to allow for the strain of subsequent grinding. The specimen thus mounted is thinned by grinding. This is done manually and the process is both time-consuming and tedious. The slide is held between the finger and thumb and with a brisk to-and-fro movement on a piece of carpenter's sandpaper (No.$1\frac{1}{2}$) the section is ground down to about half the original thickness. The grinding is then continued on a finer piece of sandpaper (No.0) and with a gentler and slower movement the section can be ground to a thickness of about 1/80th of a millimeter (Fig. 11).

Now the bone section is too thin for any more grinding on sandpaper and further grinding is carried on on a series of glass slabs each measuring 5" x $2\frac{1}{2}$" x 1". Three of the four slabs are previously "grained" in a descending order of coarseness by carborundum powder of different grades and the grains are checked microscopically. The first slab contains about a hundred grains per square centimeter; the /

the second, fifty; the third, ten, and the last is a plain, smooth glass slab which is sprinkled with a pinch of diamantine powder. Extreme care is imperative during the eight-and-a-half hours' average grinding on these slabs. The slabs must be scrupulously clean before taking the specimens on them as any grit is liable to crumble the bone during grinding. Use of any lubricant during grinding is unnecessary and should be avoided as the lubricant coming in contact with bone-dust forms sticky grits which produce artefacts during subsequent microscopical examination and may also sometimes crumble the bone during the process of grinding. A specially-designed slide-holder is used at this stage as the specimen has become by now too thin for any effective grinding by holding the slide between the finger and thumb (Figs. 9, 10 and 12).

The specimen is ground on each slab for about two hours exerting an even pressure and making not more than fifty to-and-fro movements per minute. Care is taken not to exert any lateral tilt to the slide as it is apt to crumble the underlying bone. The polishing is done on the plain glass slab sprinkled with a pinch of diamantine powder.

Washing the Specimen

It is necessary to clean the specimen and also the surrounding area of the slide before taking it for counting and autoradiography as any bone dust containing /

containing radioactive element left over after grinding will activate the photographic emulsion and thus vitiate our observation. Absolute alcohol is used for this purpose as water is liable to dissolve the calcium phosphate molecules in the bone. After rinsing the slide in absolute alcohol for ten to fifteen minutes, the top of the section is gently brushed with an ordinary camel-hair brush dipped in absolute alcohol and finally, the alide is dried by blowing air from an electric fan.

Experiment 2.- Fifty young rats, between 34 and 43 g. in weight, were taken just after weaning. They were divided into two equal groups. Rats in Group A were maintained on normal diet and surroundings and those in Group B were maintained in a covered cage kept in the dark and on the following rachitogenic diet:-

Whole yellow maize, ground	-	76 per cent
Ground gluten	-	20 per cent
Calcium carbonate	-	3 per cent
Sodium chloride	-	1 per cent

The diet was given in the form of semi-solid paste made with salt-free redistilled water. During the daily meals, the rats were given the same salt-free water to drink.

The animals were thus maintained for four weeks and the X-rays of the skeleton taken showed the appearance /

appearance of rickets in bones (Fig. 4) which was later confirmed by histological examination. Twelve rats from each of the two groups were taken and after taking the individual weights, the animals were ear-marked for subsequent identification. Each of the twenty-four rats was then injected intraperitoneally with 50 microcuries of radioactive sodium ortho-phosphate solution contained in 1 ml. of normal saline. It took twenty-four minutes to complete the injection, following which the animals were sacrificed at hourly intervals, killing two rats every hour, one from the rachitic group and the other from the normal group serving as control. The femurs were dissected out, cleaned and dehydrated. After taking the individual dry-weights, the specific activity for each bone was counted in the Geiger-Müller counter. Thin sections of bone were obtained in the same way as in Experiment 1, which were subsequently examined histologically and autoradiographed. The experiment was repeated once in order to verify the results.

Experiment 3.- Sixty-one healthy, adult rats, six to eight weeks old, and between 130 - 200 g. in weight, were divided into two groups and maintained on a uniform, normal diet.

In Group A, thirty-six animals were divided into twelve sub-groups having three rats in each sub-group. Two of the three were injected intra-peritoneally with twenty-five Collip units of para-thormone and the other kept as control. One of the two /

two injected rats had an additional intraperitoneal injection of 50 microcuries of radioactive sodium orthophosphate in 1 ml. of normal saline for subsequent autoradiogram of the femurs. The animals were killed at hourly intervals and the femurs of each rat dissected out in the same way as in Experiment 1. The bones were collected in separate, labelled bottles, containing absolute alcohol. After taking their dry-weights, the bones of the control rat, together with those of the rat injected with parathormone only, were separately estimated for total phosphorus according to the method to be described later.

The twenty-five rats in Group B were divided into five equal sub-groups. Two animals in each subgroup served as controls, while the remaining three had a daily intraperitoneal injection of twenty-five Collip units of parathormone. One of the three injected rats received an additional injection of radioactive phosphorus twenty-four hours before killing, for autoradiogram of femurs. Each group of animals was sacrificed every two days so as to cover ten days before all the rats were killed. The femurs of the two control rats as well as those of the two having parathormone alone were separately estimated for total phosphorus.

The estimation of total phosphorus in bone was carried out according to the following method:-

(a) /

(a) Bones dissolved in 2.5 ml. of 60 per cent perchloric acid ($HClO_4$).

(b) Digested for approximately two hours with occasional addition of H_2O_2 in order to remove last traces of carbon as CO_2.

(c) The digest is diluted with distilled water and made up to 250 ml.

(d) 0.3 ml. of the solution is taken in a 25 ml. flask and to it is added 1 ml. of 10 N H_2SO_4 + 2 ml. of 2.5 per cent aqueous solution of ammonium molybdate + 1 ml. of 1 amino-2 naphthol-4 sulphonic acid. The solution is made up to 25 ml. with distilled water, mixed thoroughly and left for 30 minutes. The colour-index of the solution is read on a "Spekker" colorimeter, against water as blank. From the "Spekker" reading, the amount of total phosphorus is obtained from a standard graph.

Details of preparation of bone specimens for counting the specific activity of the bones of animals injected with radioactive phosphorus are as follows: Each femur was incinerated by placing it on a sheet of wire gauze over a bunsen burner for about fifteen minutes. The incinerated, brittle bone was then gently transferred into a mortar and thoroughly powdered. The total ash content of each bone was then divided into small amounts and evenly spread out on a planchette in a very thin layer. The amount on each planchette was kept within /

within 5 mg. so as to keep the self-absorption coefficient to a minimum. Before putting it into the lead chamber of the Geiger-Müller counter, the top of each planchette was covered with a thin collodion film-absorber. The specific activity was then measured in the usual way.

RESULTS /

RESULTS

(1) In the normal bone, there is a rapid rise in the level of radioactive phosphorus within six hours after injection (Table I), after which the amount of phosphorus is maintained at a steady level for the remaining six hours (Curve 1), necessary adjustment being made for the decay factor.

(2) The specific activity of radioactive phosphorus in bone gradually diminishes from the second day onwards (Curve 2) and on the twenty-second day, about 20 per cent radioactive phosphorus is left in bone (Table II).

(3) In the growing bone the amount of radioactive phosphorus deposited in the metaphysis is much greater than that either in the diaphysis or epiphysis. The specific activity per milligram of bone in the metaphysis is more than three times that in the diaphysis and about twice that in the epiphysis (Table III).

(4) In induced rickets, the uptake at one hour as well as the uptake in twelve hours is greater than that in the normal bone (Curve 4, Table IV). A maximum rise of about 60 per cent is obtained within six hours, after which a steady state is reached. There is a greater variation in the uptakes of phosphorus in the rachitic than in the normal bone, as indicated by the greater altitudes in the rachitic uptake /

uptake curve (Curve 4).

(5) With twenty-five Collip units of parathyroid extract, very little mobilisation of phosphorus takes place. Only about 10 per cent of phosphorus is mobilised within two days after injection and there is no further fall in the phosphorus level during the following eight days (Table V).

(6) In the autoradiograms of the normal as well as of the rachitic bones, using the Pelc technique, a gradually-increasing density of the bone shadows is obtained with longer exposures (Figs. 22 - 30). The minimum exposure required to obtain a clear image is found to be twelve days, both for the normal (Fig. 27) as well as for the rachitic bone (Fig. 44). The greater deposition of radioactive phosphorus in rachitic bones is evident from their deeper autoradiograms (Figs. 38 - 44). A control autoradiogram was done with each series, in order to eliminate the artefacts as well as mechanical pressure marks.

DISCUSSION /

DISCUSSION

The maximum deposition of radioactive phosphorus in the normal adult rat femur takes place within six hours after an intravenous or intraperitoneal injection. This finding agrees with that of Manly and Bale (1939), although in their retention curve, the rate of deposition during the first six hours has been shown to be quicker and the ascent steeper, but in view of the fact that normal bone tissue takes thirty days (Hevesy, 1937) or more (Manly and Bale, 1939) completely to eliminate the injected phosphorus, it is probable that the rate of deposition in bone will also be relatively slower.

The time taken by bone to eliminate 20 per cent of the injected radioactive phosphorus is found to be twenty-two days (Curve 2). Hevesy (1923) found that within one month after injection, the total amount of the injected phosphorus is eliminated from the bone. Manly et al. (1940) and Cohn and Greenberg (1939) have also found similar results in their experiments.

The greater deposition of radioactive phosphorus in the metaphysis of a growing long bone is only to be expected, considering its greater vascularity and the greater demand on phosphorus by a growing end. The level of phosphorus in the metaphysis comes down in /

in the second week to less than half of the earlier reading. This confirms and extends the work of Manly and Bale (1939) who found nearly a 25 per cent increase in the metaphysis on the seventeenth day. The values for the diaphysis remain more or less constant throughout these four weeks of epiphyseal loss of phosphorus and this is due probably to either a greater dynamic flux in the epiphysis (Moore, 1948) or an actual transference of diaphyseal phosphorus from its labile fraction to the stable fraction (Manly et al., 1940).

In the rachitic bone, the twelve hour uptake of phosphorus is nearly three times that in the normal bone, the maximum deposition being at the sixth hour. The greater variation in the uptake in the rachitic series is due probably to greater dynamic flux in rachitic bone.

There is no significant macroscopic evidence of phosphorus mobilisation in bone after administration of twenty-five Collip units of parathyroid extract for ten days although by biochemical examination a difference of 10 per cent in the total inorganic phosphorus content is found between the normal and the bone treated with parathyroid extract. It appears from this observation that with twenty-five Collip units of parathormone circulating in the blood stream, it /

it takes more than ten days for the bone to show any obvious clinical manifestation of osteitis fibrosa cystica.

The autoradiographic studies* show obviously a greater intensity in the bone shadows with longer exposures, and with 50 microcuries of radioactive phosphorus an exposure of twelve days is necessary to obtain a clear outline of the femur. The chief drawback to exposing the specimens for longer time periods, which is necessary with a smaller dose of the radioactive isotope, is the lack of contrast owing to the partial development of the surrounding grains by prolonged β-radiation. It is obvious from Figs. 26 and 30 that an increased dosage (200 - 300 microcuries) of the radioactive isotope will produce a sharper and clearer autoradiogram by cutting short the exposure-time and thus preventing the surrounding grains from being developed by the residual emanations. Moreover, artefacts, indistinguishable from developed grains, are less likely to show up and vitiate the background when the specimen is exposed for a shorter length of time by using a relatively higher concentration of the radioactive isotope.

SUMMARY AND CONCLUSIONS /

*Figs. 22 - 30 and 38 - 44 are autoradiograms of specimens similar to those discussed in the text. In some of the illustrations the background has been obliterated for the sake of better contrast and sharpness of image.

SUMMARY AND CONCLUSIONS

(1) A study of the metabolism of phosphorus in bone has been carried out in albino rate with radioactive phosphorus in the normal as well as in induced pathological conditions and the results have been compared.

(2) It is found that in the normal as well as in induced rachitic rats, the maximum uptake of marked phosphorus takes place within six hours after injection. The level of concentration and the rate of deposition of radioactive phosphorus have been found to be higher in rachitic bones than in the normal and 20 per cent of the injected phosphorus has been found to be eliminated from bone in twenty-two days.

(3) Using twenty-five Collip units of parathyroid extract, only 10 per cent phosphorus is mobilised from bone in two days and with the same dosage, negligible variation in the total phosphorus content of bone is observed in twelve hours.

(4) Autoradiographic studies have been undertaken in normal and in induced pathological bones and a method has been described for obtaining thin sections of undemineralised bone. Autoradiograms of bone have been obtained by using a classical technique and a modification of that technique is described.

The /

The minimum exposure required to obtain clear autoradiograms of a thin section of rat femur is also mentioned.

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TABLE I

Uptake of Radioactive Phosphorus in Normal Rat Femurs during Twelve Hours. Dose - 150 microcuries

Time after injection in hours	Total activity	Weight of femur in mg.	Specific activity per mg. bone
1	12629	455	27
2	20061	505	39
3	17201	475	36
4	20038	450	44
5	19106	415	46
6	21162	405	52
7	14421	290	49
8	20702	460	45
9	22296	480	46
10	20460	425	48
11	20702	460	45
12	14421	290	49

TABLE II

Rate of Elimination of Radioactive Phosphorus in Normal Rat Femurs during Twenty Two Days. Dose - 150 microcuries

Time after injection in days	Specific activity	Decay factor in %	Specific activity corrected for decay
4	2730	35	7800
6	3431	38	9028
8	2849	42	6547
10	2969	46	6672
12	2424	52	4661
14	2441	56	4359
16	1925	62	3105
18	1795	68	2639
20	1631	75	2174
22	1303	82	1588

TABLE III

Growing Bone: Normal:

Specific Activity of Radioactive Phosphorus in Metaphysis, Epiphysis and Diaphysis of Rat Femurs on the Seventh Day after Injection of 150 microcuries of Radioactive Phosphorus

Specimen No.	Metaphysis			Epiphysis			Diaphysis		
	Weight in mg.	Total activity	Specific activity per mg.	Weight in mg.	Total activity	Specific activity per mg.	Weight in mg.	Total activity	Specific activity per mg.
1	25	3525	141	38	3230	85	50	2355	47
2	45	6352	141	42	3864	92	100	4703	47
3	32	4475	140	35	2905	83	70	3290	47
4	42	5990	142	40	3520	88	97	4463	46

TABLE IV

Induced Rickets: Uptake of Radioactive Phosphorus by Rat Femurs during Twelve Hours
Dose - 150 microcuries

Time after injection in hours	Total activity	Weight of femur in mg.	Specific activity per mg. bone
1	19488	224	87
2	14346	170	84
3	20595	200	103
4	15587	140	111
5	14350	120	119
6	14480	110	131
7	23700	205	115
8	17894	170	105
9	16410	150	109
10	22366	211	106
11	29250	265	110
12	19110	182	105

TABLE V

Mobilisation of Phosphorus in Rat Femur during Ten Days after Injection of 25 Collip Units of Parathormone Extract

Time after injection in days	No. of femurs removed	Total phos-phorus in mg.	Total phos-phorus per femur in mg.
2	2	39.4	19.7
4	2	35.6	17.8
6	2	35.0	17.5
8	2	35.6	17.8
10	2	35.2	17.6

Fig. 1.- Albino rat. Four weeks old.
Normal diet. Weight 175 gm.

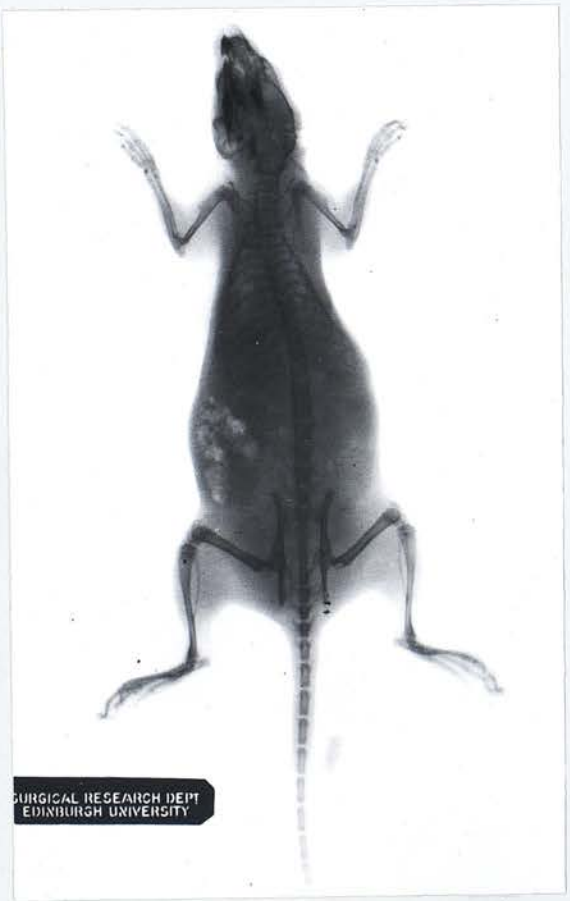


Fig. 2.- X-ray of rat
shown in Fig. 1.

Fig. 3.- Albino rat from same stock as Fig. 1.
Four weeks old. Rachitogenic diet.
Weight 140 gm.

Fig. 4.- X-ray of rat
shown in Fig. 3.

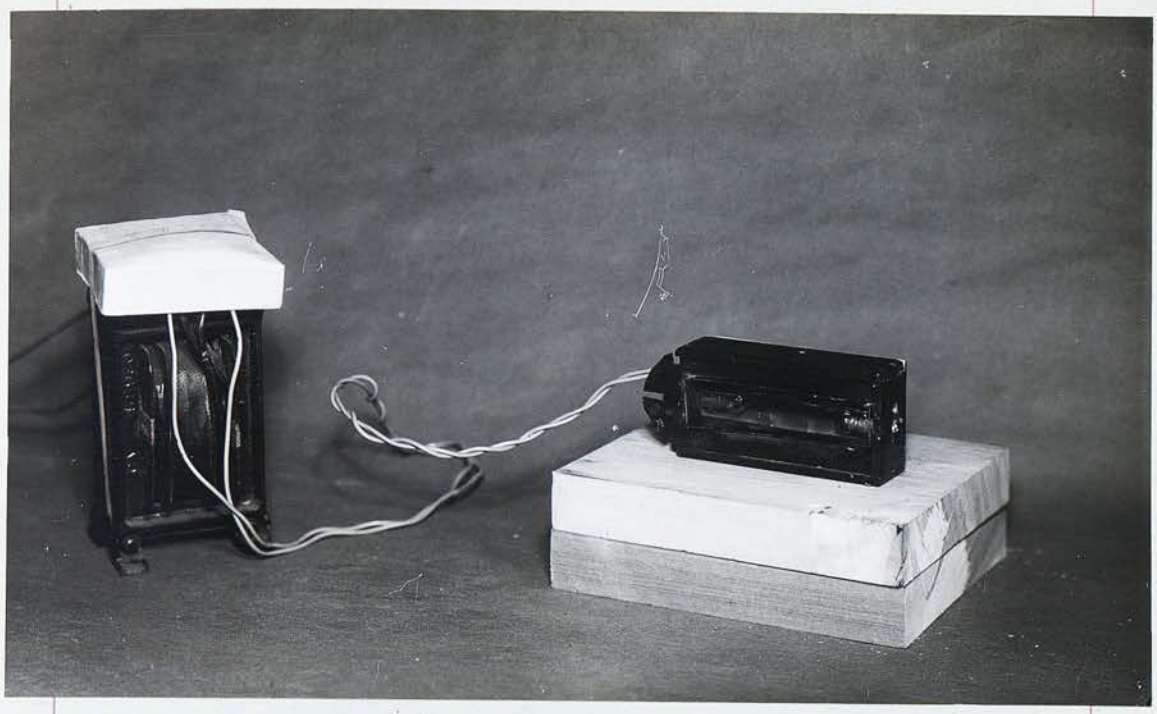


Fig. 5.- Showing slit lamp connected with electric cell used for transillumination of tail vein of rat.

Fig. 6.- Showing apparatus and method of injecting tail vein of rat.

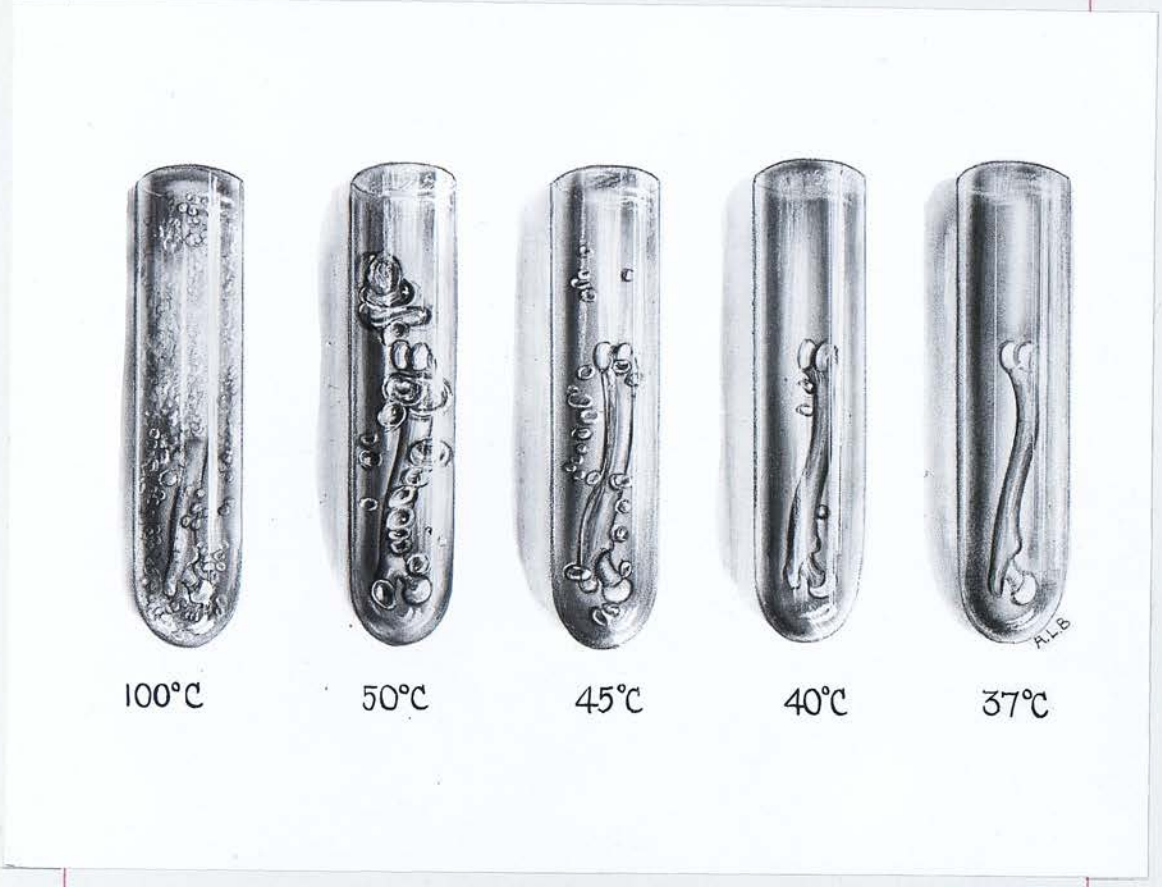


Fig. 7.- Showing specimens of rat femur mounted in liquid perspex. Note temperature for complete elimination of air bubbles.

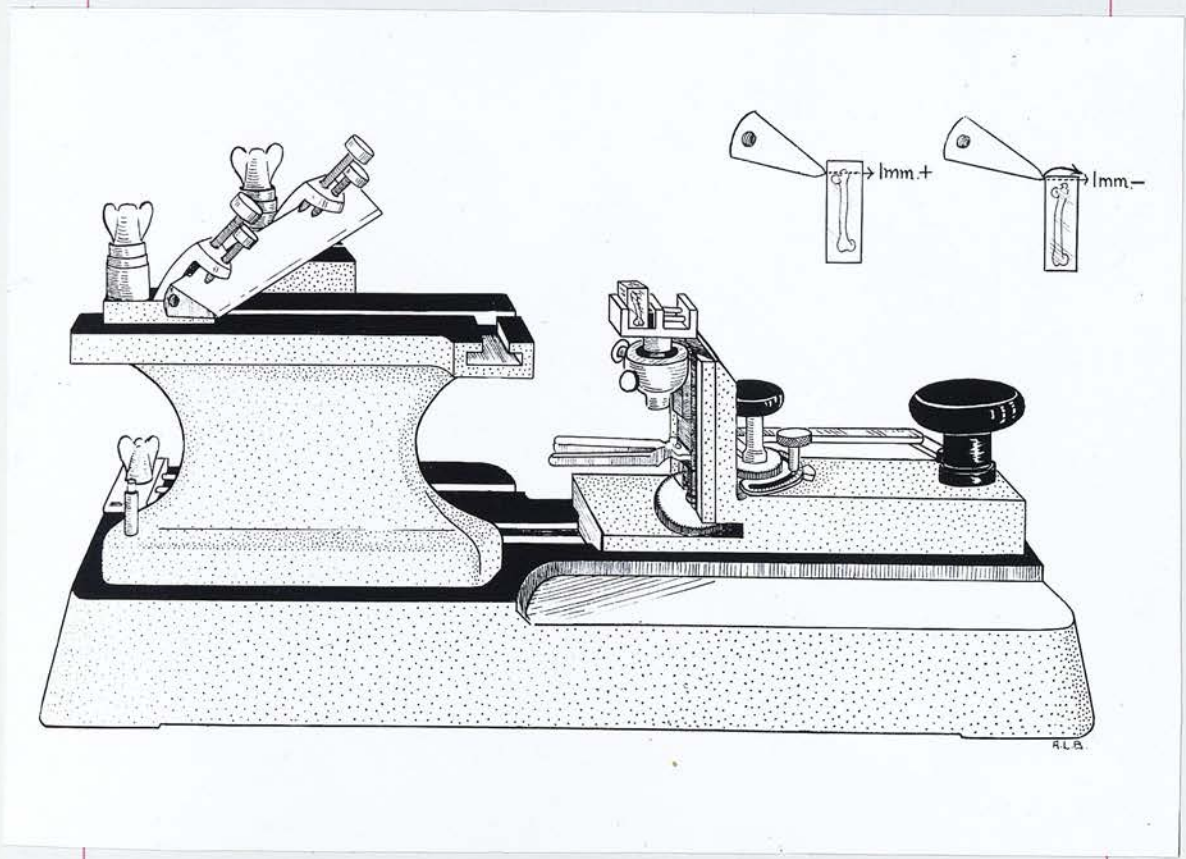


Fig. 8.- Heavy sledge precision microtome (M.S.E.) used for cutting section through undecalcified bone.

Insets.- Showing how the knife slips over if the specimen is less than 1 mm. in thickness.

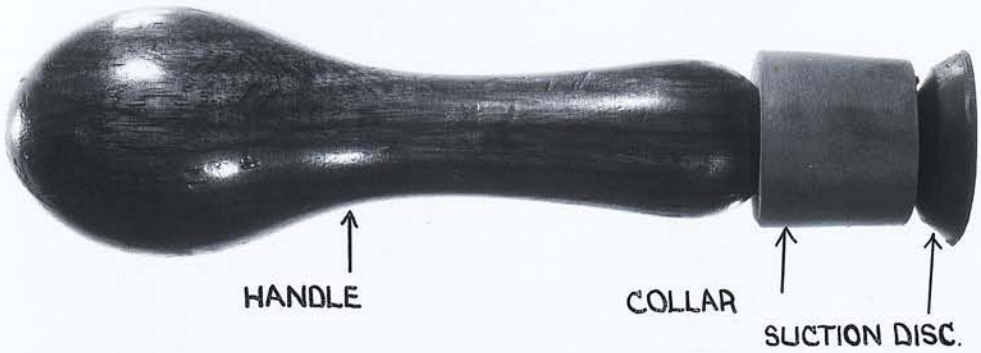


Fig. 9.- Showing the handle used to hold mounted perspex slide for grinding bone.

Fig. 10.- Showing the grinding handle in use.

Fig. 11.- Apparatus employed for bone grinding.

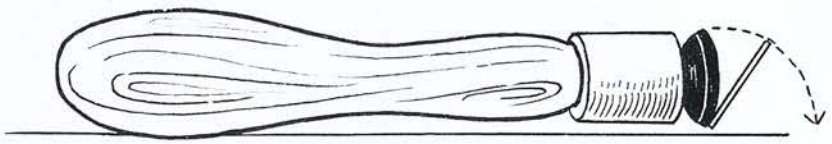


Fig. 12.- Showing automatic release of slide from the grinding handle due to decompression of suction disc.

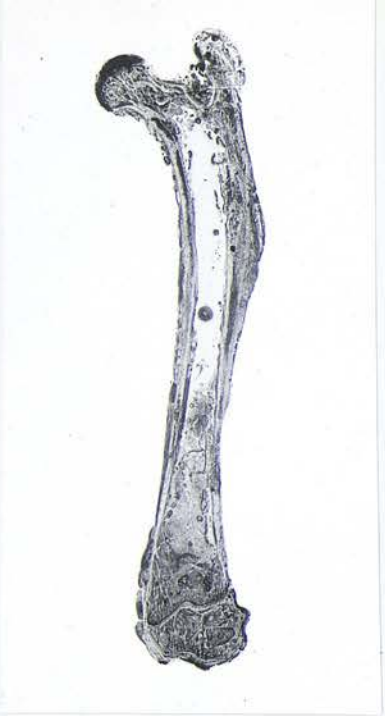


Fig. 13.- Longitudinal section of rat femur. (Low power). 8 microns: (straight photograph).

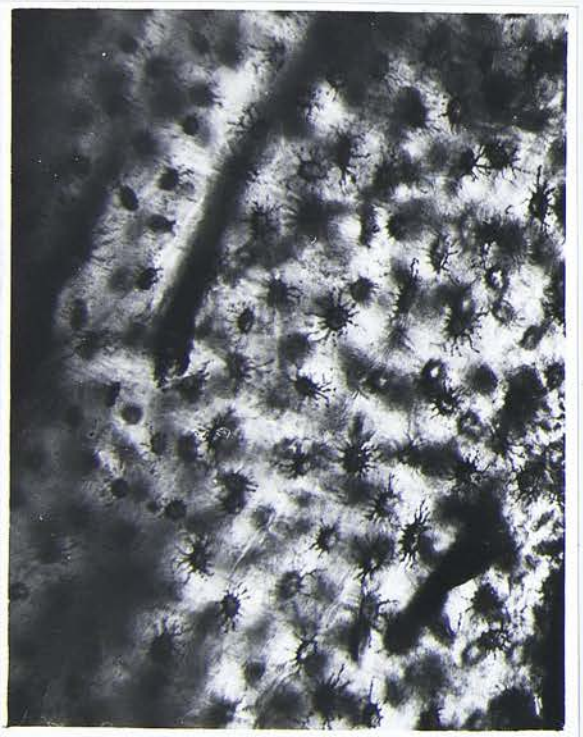


Fig. 14.- Transverse section of rat femur showing the osteocytes. Phase contrast (X250). 5 microns: (straight photograph).

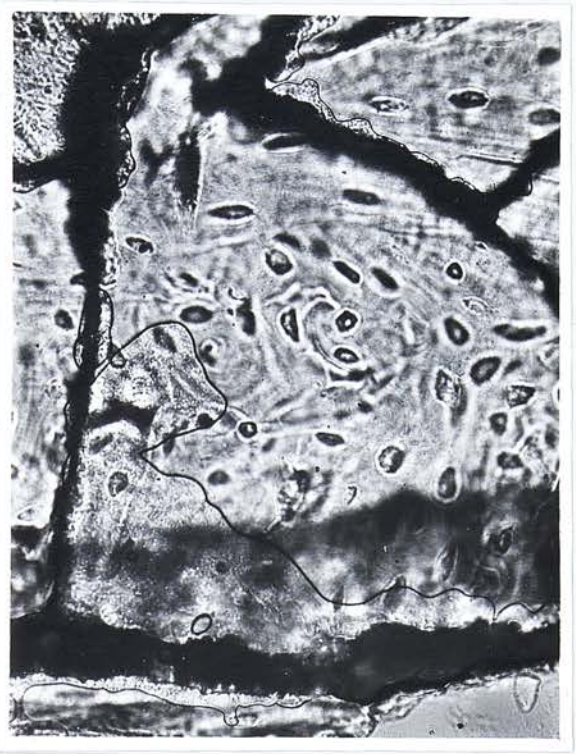


Fig. 15.- Transverse section of rat femur showing the clear "capsule" of osteocytes. Phase contrast (X400). 5 microns: (straight photograph).

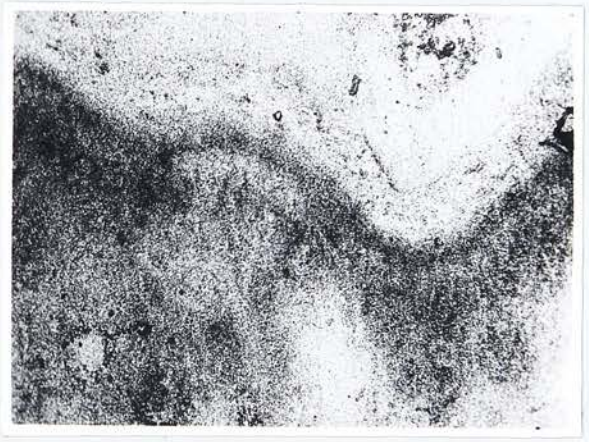


Fig. 16.- Showing the epiphyseal line in the growing end of a rat femur. Phase contrast (X60): (straight photograph).

Fig. 16A.- Longitudinal section of normal rat femur before injection of radioactive phosphorus (control specimen). (5 - 8 microns): (straight photograph)

Fig. 16B.- Autoradiogram of Fig. 16A. 12 days.

Fig. 16C.- Longitudinal section of induced rachitic rat femur before injection of radioactive phosphorus (control specimen). (5 - 8 microns): (straight photograph)

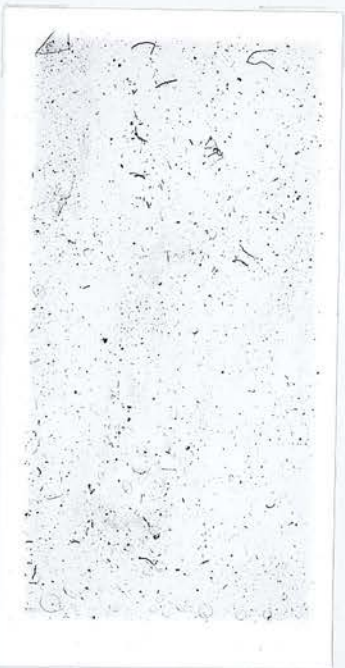


Fig. 16D.- Autoradiogram of Fig. 16C. 12 days.

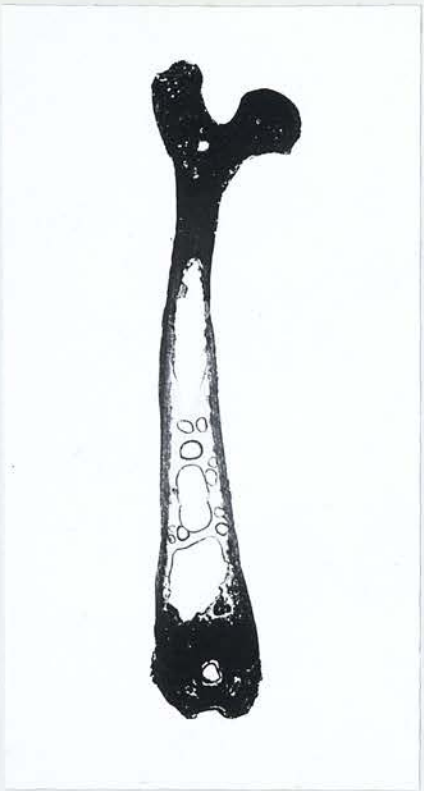
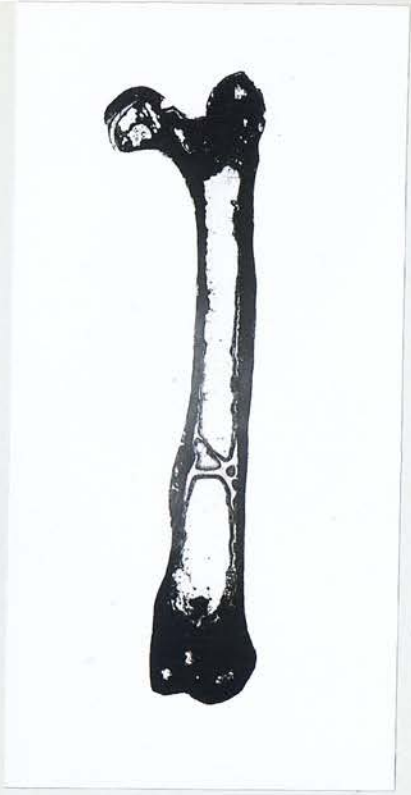


Fig. 17.- Longitudinal section of rat femur injected with radio-active phosphorus. Normal: (straight photograph).

Fig. 18.- Longitudinal section of rat femur injected with radio-active phosphorus. Normal: (straight photograph).



Figs. 19 and 20.- Longitudinal sections of rat femur injected with radioactive phosphorus. Normal: (straight photograph).

Fig. 21.- Longitudinal section of rat femur injected with radioactive phosphorus. Normal: (straight photograph).

Fig. 22.- Autoradiogram of rat femur (4-8 microns) 50 microcuries. 2 days. Normal.

Fig. 23.- Autoradiogram of rat femur (4-8 microns) 50 microcuries. 4 days. Normal.

Fig. 24.- Autoradiogram of rat femur (4-8 microns) 50 microcuries. 6 days. Normal.

Fig. 25.- Autoradiogram of rat femur (4-8 microns) 50 microcuries. 8 days. Normal.

Fig. 26.- Autoradiogram of rat femur (4-8 microns) 200microcuries. 4 days. Normal.

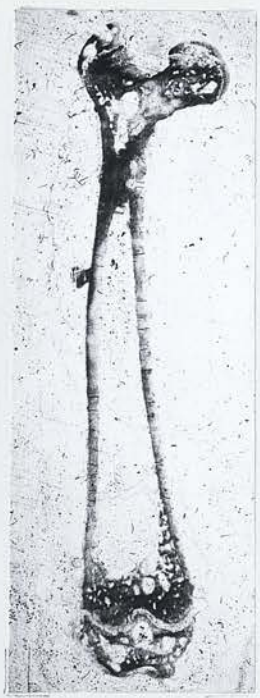


Fig. 27.- Autoradiogram of rat femur (4-8 microns) 50 microcuries. 12 days. Normal.

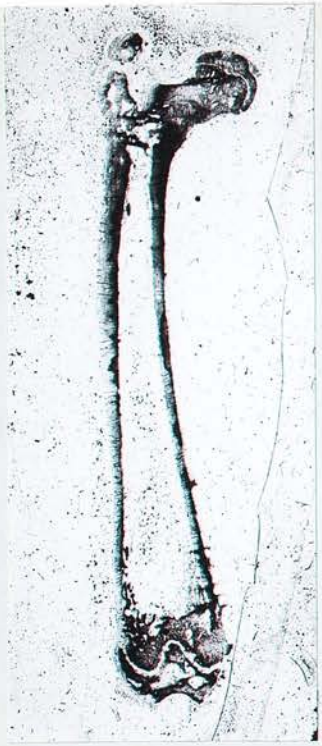


Fig. 28.- Autoradiogram of rat femur (4-8 microns) 50 microcuries 12 days. Normal.

Fig. 29.- Autoradiogram of rat femur (4-8 microns) 50 microcuries 14 days. Normal.

Fig. 30.- Autoradiogram of rat femur (4-8 microns) 300 microcuries 6 days. Normal.

Fig. 30A.- Autoradiogram of rat femur (5-8 microns) 50 microcuries 4 days. Normal.

Fig. 30B.- Autoradiogram of rat femur (5-8 microns) 50 microcuries 8 days. Normal

Fig. 30C.- Autoradiogram of rat femur (5-8 microns) 50 microcuries 12 days. Normal.

Fig. 31.- Longitudinal section of rachitic rat femurs injected with radiophosphorus: (straight photograph).

Fig. 32.- Longitudinal section of rachitic rat femurs injected with radiophosphorus: (straight photograph).

Fig. 33.- Longitudinal section of rachitic rat femurs injected with radiophosphorus: (straight photograph).

Fig. 34.- Longitudinal section of rachitic rat femurs injected with radiophosphorus: (straight photograph).

Fig. 35.- Longitudinal section of rachitic rat femurs injected with radiophosphorus: (straight photograph).

Fig. 36.- Longitudinal section of rachitic rat femurs injected with radiophosphorus: (straight photograph)

Fig. 37.- Longitudinal section of rachitic rat femurs injected with radiophosphorus: (straight photograph).

Fig. 38.- Autoradiogram of rachitic rat femur.
4.8 microns.
2 days.

Fig. 39.- Autoradiogram of rachitic rat femur.
4.8 microns.
4 days.

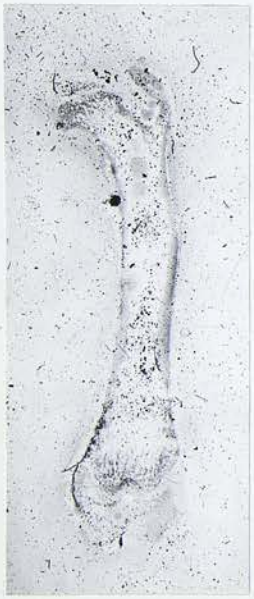


Fig. 40.- Autoradiogram of rachitic rat femur.
4.8 microns.
8 days.

Fig. 41.- Autoradiogram of rachitic rat femur.
4.8 microns.
9 days.

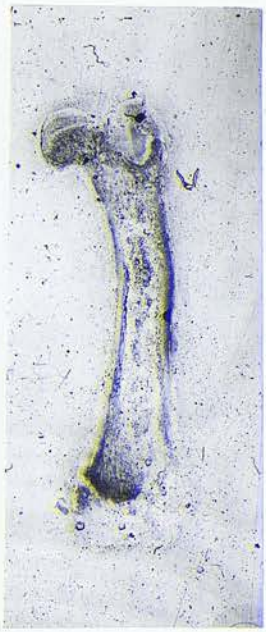


Fig. 42.- Autoradiogram of rachitic rat femur.
4.8 microns.
10 days.

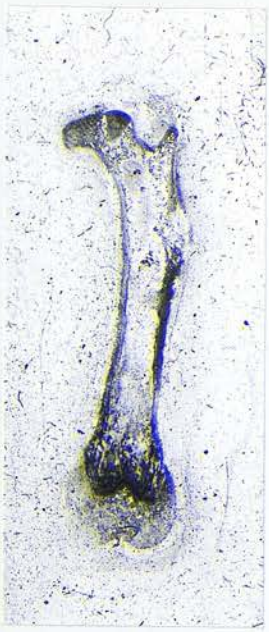


Fig. 43.- Autoradiogram of rachitic rat femur.
4.8 microns.
11 days.

Fig. 44.- Autoradiogram of rachitic rat femur.
4.8 microns.
12 days.

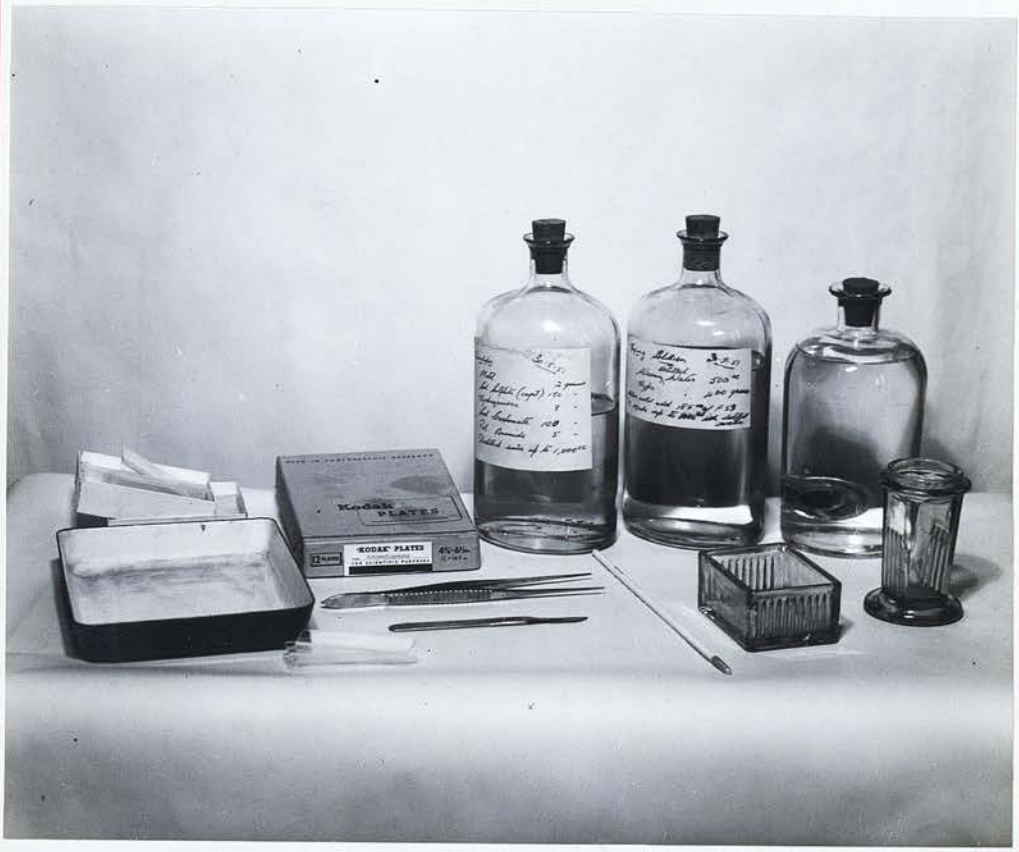


Fig. 45.- Equipment for bone autoradiography:

1. Developing solution
2. Fixing solution
3. Redistilled water
4. Koplin jar
5. Slide tray
6. Thermometer
7. Scalpel
8. Fine pointed forceps
9. Perspex slides
10. Enamel tray
11. Glass slides
12. Kodak nuclear track emulsion plates

Fig. 46.- Showing the steps for bone autoradiography.

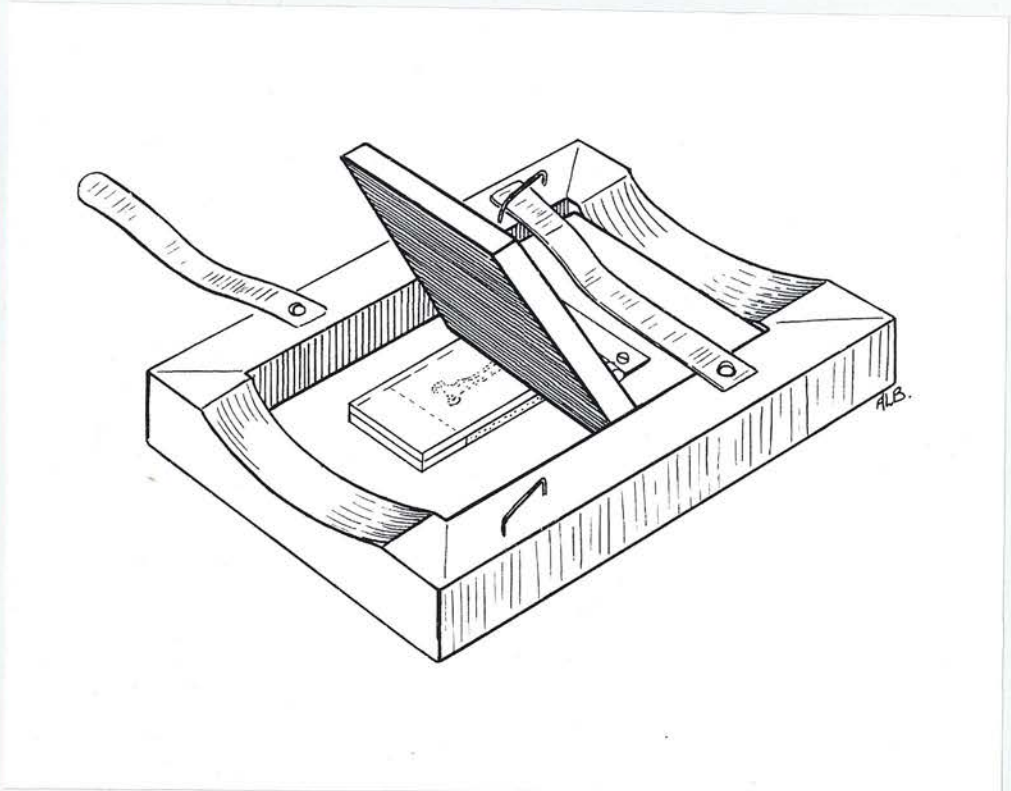
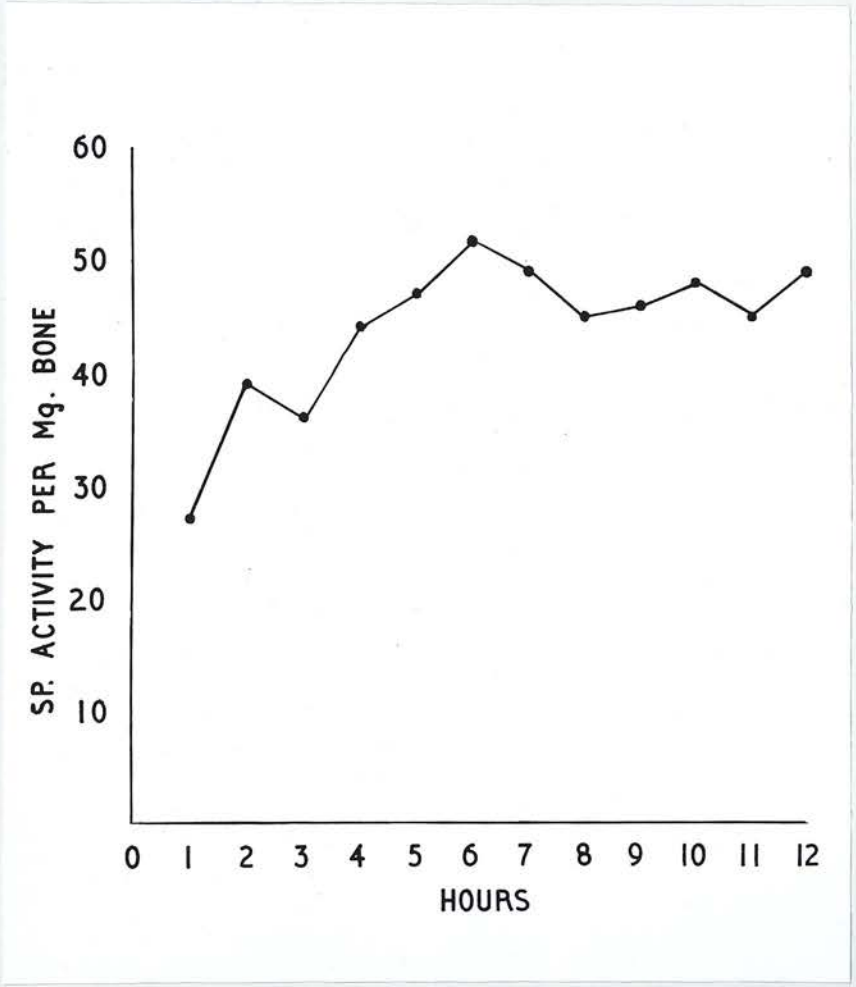
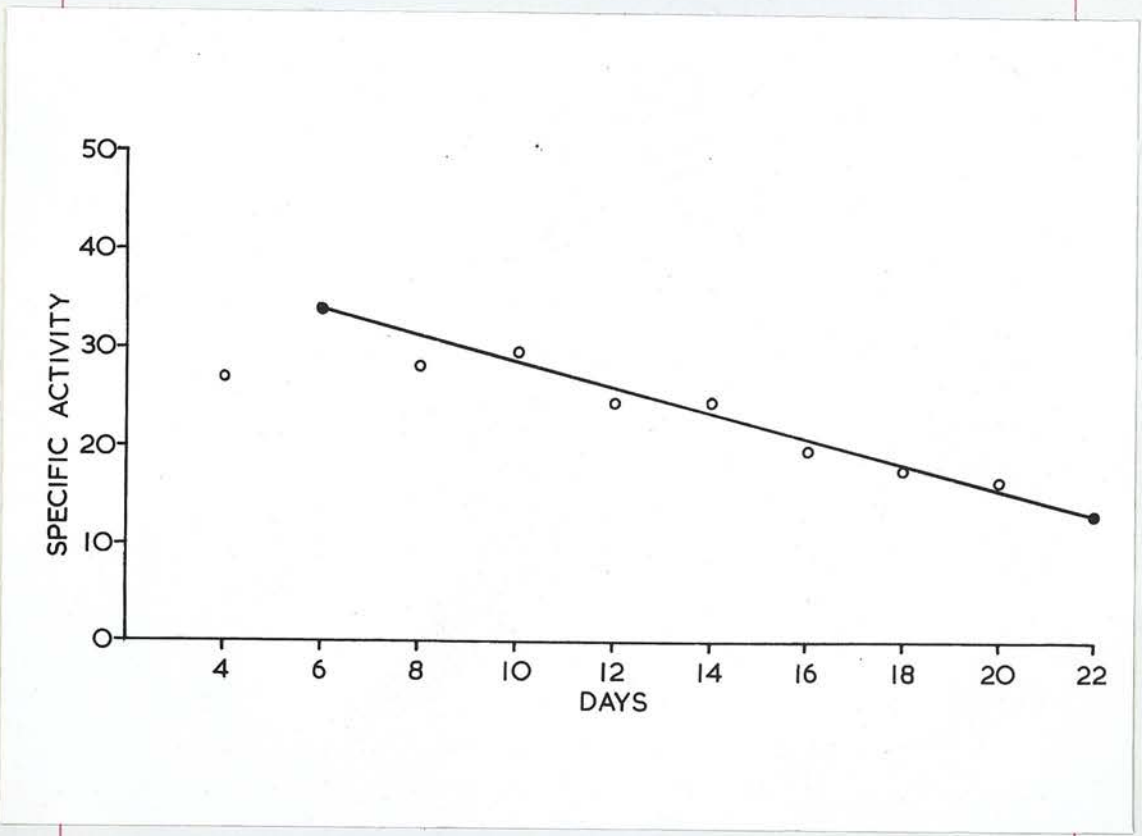


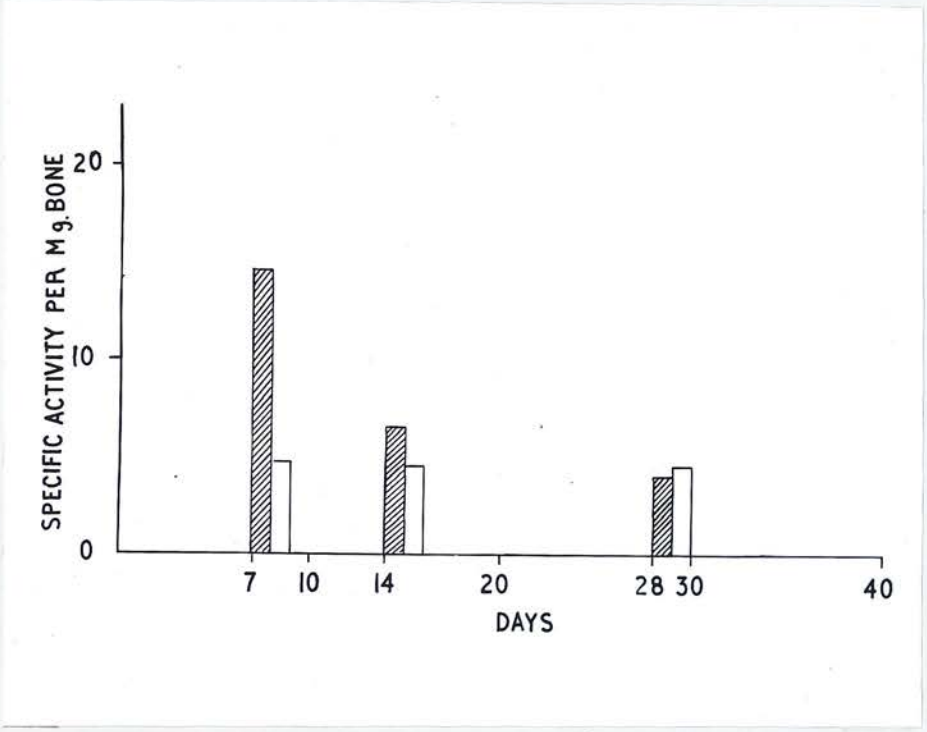
Fig. 47.- Showing final mounting in a photographic contact frame for bone autoradiography.



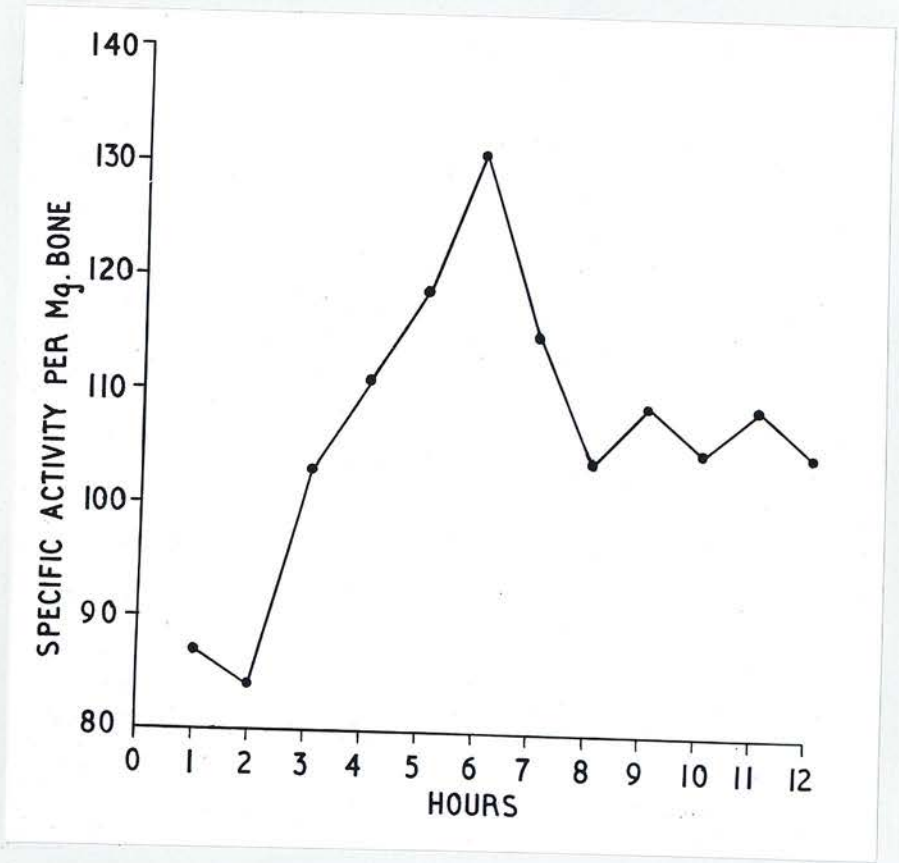
Curve 1.- Showing uptake of radiophosphorus by rat femur in twelve hours. 50 microcuries.



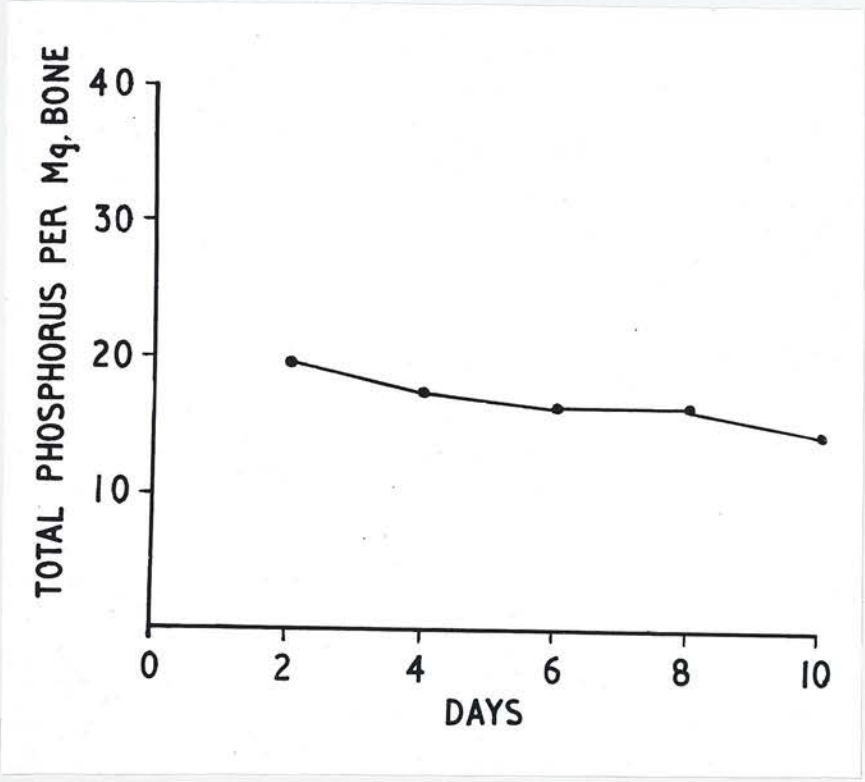
Curve 2.- Showing rate of elimination of radioactive phosphorus from rat femur.



Curve 3.- Showing the relative deposition of radiophosphorus in the epiphysis and diaphysis of rat femur. 50 microcuries. Hatched area represents epiphyseal deposition.



Curve 4.- Showing uptake of radiophosphorus by rachitic rat femur in twelve hours. 50 microcuries.



Curve 5.- Showing the extent of mobilization of phosphorus from bone in ten days in rats treated with parathyroid extract.